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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Inventors	:	Britta Hardy and Avraham Novogrodsky
Serial No.	:	08/380,857
Filed	:	January 30, 1995
Title	:	*Immuno-Stimulatory Monoclonal Antibodies*
Group Art Unit	:	1806

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DECLARATION

Honorable Commissioner of Patents and Trademarks

SIR:

I, Dr. Britta Hardy, do hereby declare that:

1. I am one of the inventors for Patent Application No. 08/380,857 (hereinafter: "*the application*").

2. I am the head of the Cellular Immunology Research Unit at the Felsenstein Medical Research Center, Rabin Medical Center, Belinson Campus, Israel (affiliated with the Tel Aviv University, Sackler School of Medicine). My curriculum vitae and the list of my publications are attached hereto as Enclosure "**A**" and "**B**", respectively.

3. Below are results of several experiments which were performed by me and under my supervision. The results of these experiments provide further evidence that the anti-B lymphoblastoid monoclonal

antibody (BAT-mAb) of the invention possess anti-tumor activity and are effective as anti-human-derived tumor agents.

In earlier experiments carried out in accordance with the invention and disclosed in the application, the anti-tumor effect of the BAT-mAb was demonstrated in mice bearing various murine tumors. A single intravenous administration of the BAT mAb to mice inoculated with B-16 melanoma cells, 3LL Carcinoma Cells, or MCA fibrosarcoma cells, resulted in tumor regression as well as in the prolonged survival of the tumor bearing mice.

The present results, carried out in accordance with the invention and with the BAT mAb of the invention as described below, show the anti-tumor activity of the BAT mAb in mice bearing human tumors and demonstrate the potential of this antibody as an anti-tumor agent in humans.

4. In the following, the anti-tumor activity of the BAT mAb will be demonstrated in nude mice carrying human tumor xenografts and in SCID mice engrafted with human lymphocytes and inoculated with human tumor cells.

5. <u>Experimental Results</u>

5.1 **Brief Description of the Figures**:

<u>Fig. 1</u> is a graphic representation showing the tumor volume (mm^3) in nude mice injected subcutaneously (s.c.) with 10^6 cells per mouse of HT-29 Human Colon Carcinoma cells and receiving an intravenous (i.v.) injection of the BAT mAb at a concentration of 10 μg per mouse.

<u>Fig. 2</u> is a photograph showing lungs obtained from SCID mice which were inoculated with SK-28 human melanoma cells only (upper row in the photograph) and of lungs from SCID mice which were inoculated with SK-28 melanoma cells and treated with the BAT mAb (lower row in

the photograph). All the mice were engrafted with human peripheral blood monocytes (PBM).

Fig. 3 is a graphic representation showing Thymidine uptake in human Peripheral Blood Lymphocytes (PBL) which were co-cultured on HT-29 human colon cells and after one day were washed and cultured in the absence or in the presence of the BAT mAb (at a concentration of 0.1 μg/ml) for three additional days.

5.2 Materials and Methods

5.2a Generation of BAT-mAb:

BAT was generated and purified as previously described. In brief, BALB/C mice were immunized with membranes from Daudi cells. Spleen cells were fused with myeloma NS-O cells. BAT was selected by its ability to bind Daudi cells and by its ability to induce proliferation of human peripheral blood mononuclear cells (PBMC). Cells were grown in RPMI-1640 supplemented with fetal calf serum (10%), sodium pyruvate, glutamine and antibiotics and incubated at 37°C in a humidified atmosphere containing 5% CO_2. BAT was purified on a protein G sepharose column according to manufacturers' instructions (Pharmacia).

5.2b Cell preparations for engraftment to SCID mice:

PBMC were obtained from blood of healthy donors by Ficoll/Hypaque density centrifugation. Cells were washed and suspended in PBS. $5X10^7$ cells were injected i.p. to each SCID mouse in order to construct a human immune system in these mice.

5.2c Activation of lymphocytes by tumor cells and BAT

Human PBL ($2x10^6$/ml) were incubated on HT-29 human colon carcinoma cells monolayers for one day. PBL cells were then

removed from the tumor cell monolayer, washed twice with medium and suspended at the initial concentration. Splenocytes obtained from tumor-inoculated mice were suspended at $2x10^6$/ml and BAT at 0.1 *μg/ml* was added for 3 days in vitro.

5.2d **Proliferation assay of lymphocytes upon incubation with BAT**:

PBL were separated from PBMC by removing the adherent monocytes after one hour incubation on plastic petri dishes. Aliquots of $2x10^6$ PBL (200*μl*) in culture medium containing 5% human AB serum were incubated for 3 days in 96-well flat-bottom plates without and with BAT at 0.1 *μg/ml* [^{3}H] Thymidine (1*μCi/well)* was added for 20 hours before harvesting. Cultures were harvested into glass filters and radioactivity was counted using a liquid β-scintillation counter.

5.2e **Mouse tumor models**:

Five to six female mice, 6-8 weeks old were used in each group for each experiment. They were obtained from Harlan Labs. (Jerusalem, Israel) SCID mice were maintained in sterile conditions at a controlled temperature.

HT29 human colon carcinoma cells (10^6/mouse) were injected (s.c.) at the axillary region of wild type or nude mice. BAT (10 *μg*/mouse) was injected (i.p.) at day, 7,14 and 21 post-tumor inoculation. Seven days post-tumor inoculation, the size of tumor was measured daily, until day 30 post-tumor inoculation, when the untreated mice died.

SK-mel 28 (SK-28) a human melanoma derived cell line (originally obtained from Sloan-Kettering Institute, N.Y., U.S.A.) cells were injected i.v. into SCID mice at $5x10^5$/mouse. Injection of these cells resulted in tumor lesions in the lungs. SK-28 melanoma cells were inoculated one day following administration (i.p.) of anti-GM1 (35*μ*g

mouse) (Wako Chemicals, Dallas, Tx). Anti-ASGMI consists of rabbit polyclonal antibodies that recognize murine NK cells and depletes NK cells activity when injected i.p. into mice. Human PBMC ($8x10^7$/mouse) were engrafted (i.p.) to SCID mice treated with BAT by a single injection (10μg mouse) at day 14 post-tumor inoculation or on other days as indicated. Twenty-four days post-tumor inoculation the mice were sacrificed, the lungs removed, melanoma metastases were counted and the lungs' weight were determined.

6. **Results**:

<u>Experiment 1</u>:

Nude mice were implanted subcutaneously (s.c.) with human colon carcinoma cells (HT-29) as described above. The mice were divided into two groups: mice in one group received injections of the BAT mAb and mice of the other group (serving as a control) were injected with a mouse IgG_3 isotype control antibody. As seen in attached Fig. 1, the growth of the tumors in mice inoculated with the tumor cells and receiving injections of the BAT mAb was delayed up to 24 days post-tumor inoculation as compared to the control mice which did not receive the BAT mAb injections. In addition, the size of the tumor measured on day 46 post tumor inoculation in the BAT mAb treated mice was half that of the tumor size in the untreated mice on the same day.

<u>Experiment 2</u>:

SCID mice were engrafted i.p. with human PBMC and also received an i.v. inoculation of human melanoma cells (SK-28) as described above. The inoculation of human SK-28 melanoma cells led to the development of tumor lesions in the SCID mice. As seen in attached Table 1 and Fig. 2, administration of the BAT mAb to the SCID mice

engrafted with the human PBMC and inoculated with the human SK-28 melanoma cells, fourteen days after tumor inoculation, resulted in a significant regression both in the number of lung lesions in the treated mice as well as in the lung weight of these mice. Treatment of the mice with the BAT mAb as described above reduced the number of metastases in the mice' lungs from an average of 174 ± 53 to 8 ± 9 lesions in the lungs and reduced the lungs' weight in these mice from an average of 702 ± 140 mg. to a normal weight of 206 ± 70 mg. Moreover, 10 out of the 32 mice in 5 different experiments carried out as described above were completely free of tumors.

Experiment 3:

The effect of the time of administration of the BAT mAb to SCID mice engrafted with human PBL and inoculated with SK-28 melanoma cells as described in Example 2 above was tested. As seen in attached Table 2, a most significant effect of the BAT mAb was apparent when administered on day 14 post-tumor inoculation in these mice. Administration of the mAb 3 days after tumor inoculation had a marginal effect only, whereas administration 5 days and 10 days post-tumor inoculation was also effective.

Experiment 4:

The *in vitro* stimulatory effect of the BAT mAb on human PBL's that were pre-incubated on HT-29 human colon carcinoma cell monolayers was tested. As seen in attached Fig. 3, in four experiments using four human different human PBLs, the exposure of the lymphocytes to the tumor cells *in vitro*, led in itself to an increase in their proliferative response ranging from 12 to 22 fold increase in $[H^3]$ thymidine uptake. However, in lymphocytes that were pre-exposed to tumor cells and then

cultured with the BAT mAb, the thymidine uptake was significantly increased 22 to 44 fold. Lymphocytes which were incubated on allogeneic macrophage monolayers did not acquire the enhanced sensitivity to stimulation by the BAT mAb.

7. **Discussion**:

The results of the experiments disclosed in the application, including *in vitro* experiments showing the stimulatory effect of the BAT-mAb on human lymphocytes (Examples 3, 4 in the application), as well as the results of the *in vivo* experiments showing the anti-tumoral effect of the BAT-mAb against mouse tumors (Example 6-8 in the application) laid a sound basis for expecting that the BAT mAb of the invention will be effective in the treatment of human tumors as well. This is demonstrated by the above results of experiments on mice bearing tumors of human origin which were carried out, albeit after filing of the application, but in the exact manner as the experiments described in the original application, on mice bearing tumors of mouse origin. These results showed that the administration of the BAT-mAb to mice bearing tumors of human origin resulted in a significant reduction in the tumor load of the treated mice. It is to be noted that the BAT-mAb was effective in the treatment of human tumors from several different origins (human colon carcinoma cells and human melanoma cells). These results clearly indicate that the information disclosed in the original application was indeed predictive of the effect of the claimed mAb in the treatment of human tumors and cancer.

8. I further declare that all statements made herein of my own knowledge are true, that all made statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that wilful false statements and the like so made are punishable

by fine or imprisonment or both, under section 100 of Title 18 of the United States Code and that such wilful statements may jeopardize the validity of the application or any patent issued thereon.

Dated this 24th day of February 1997

B. Hardy

BRITTA HARDY

TABLE 1

Number of lung metastases and lung weight in SCID mice engrafted with human lymphocytes ,inoculated with human melanoma (SK-28) and treated fourteen days later with BAT.

	Non engrafted	Engrafted with human PBM	
BAT treatment	-	-	+
No.Lung metastases	>250	174±53	8±9
	(n=13)	(n=25)	(n=32)
Lung weight (mg.)	867±82	702±140	206±17
	(n=12)	(n=22)	(n=32)

SCID mice were injected with anti GM1 (25μg/mouse).On the following day human PBM (10^8 /mouse) were injected (i.p.). Human melanoma cells, SK-28, were inoculated (i.v.) 3-5 days later ($5\text{-}7\times10^5$ /mouse).Mice were treated with BAT (10μg/mouse) in a single injection (i.v.) 14 days post tumor inoculation. Mice were sacrificed on day 24 post-tumor inoculation and the extent of lung metastases was evaluated by scoring the number of metastases and lungs weight.

n-represents the total number of mice studied in 5 different experiments.

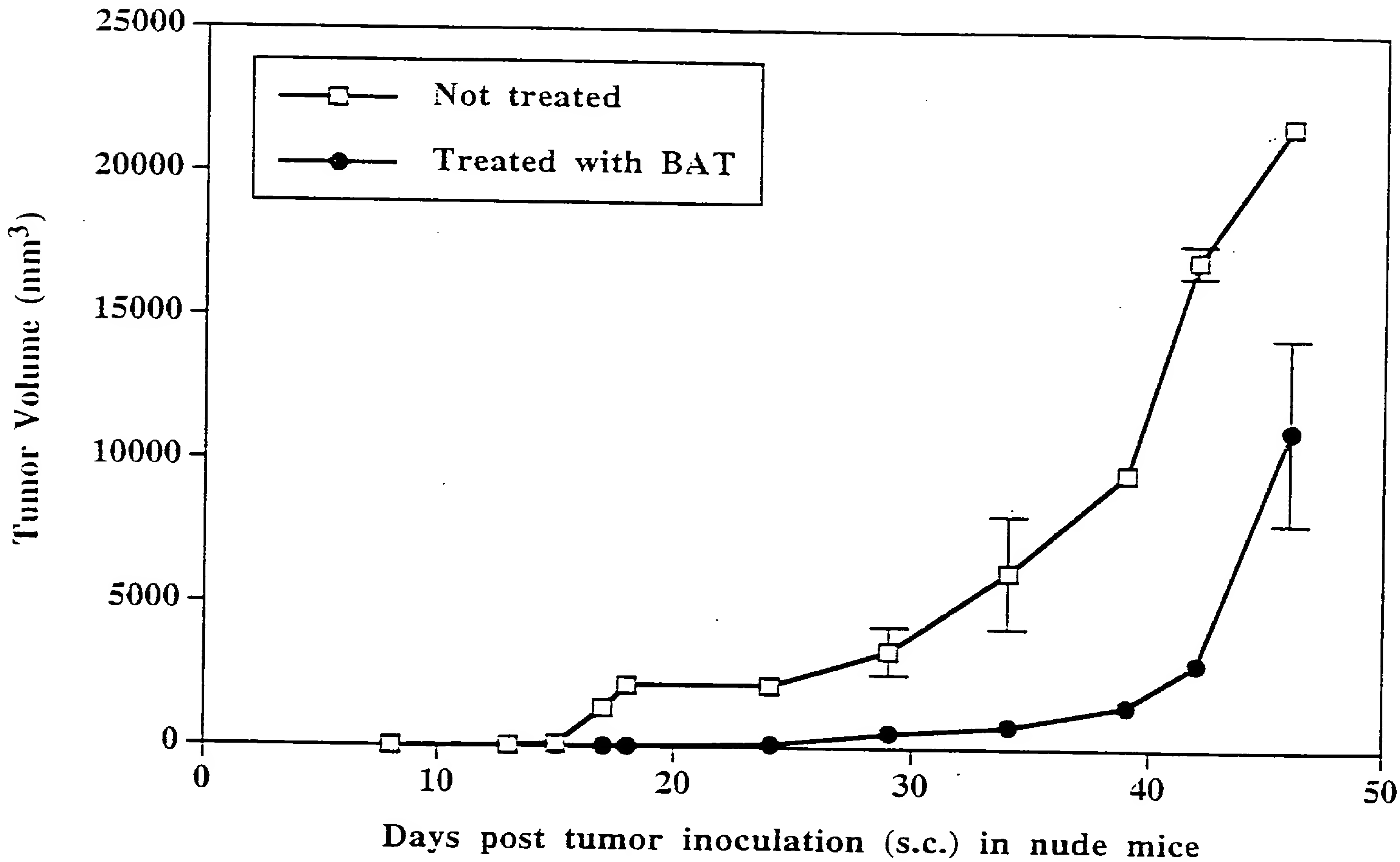
TABLE 2

BAT treatment of SCID mice engrafted with human lymphocytes at different times following human melanoma inoculation .

BAT treatment (day)	No. metastases.	Lungs' weight (mg)
No BAT	>250	750 ± 70
3	206 ± 75	665 ± 150
5	89 ± 7	315 ± 8
10	35 ± 12	219 ± 1
14	10 ± 17	217 ± 6

BAT was administered (10μg/mouse) at different time in relation to tumor inoculation at day 0. Human PBM were engrafted (i.p.) at 8.x 10^7 /cells per mouse, one day post injection (i.v.) of anti GM1 (25 μg/mouse) .Tumor cells (i.v.) at $7x10^5$ cells/mouse were injected (i.v.) 3-5 days later. 24 days post-tumor inoculation mice were sacrificed and the number of lungs` metastases and weight were determined.

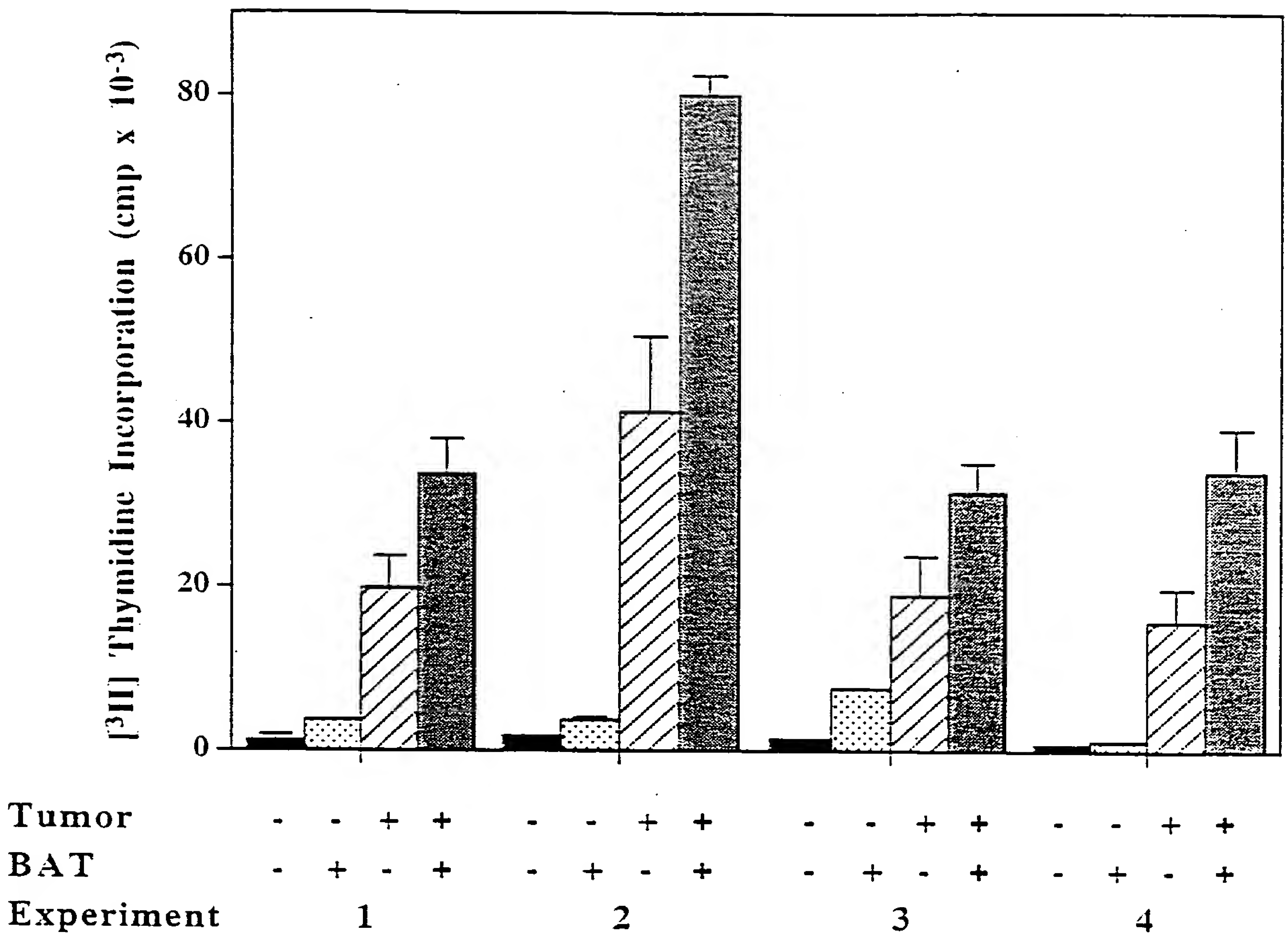
FIG. 1



25000
20000
15000
10000
5000
0
Tumor Volume (mm³)
Not treated
Treated with BAT
0
10
20
30
40
50
Days post tumor inoculation (s.c.) in nude mice

FIG. 2

FIG. 3


[3H] Thymidine Incorporation (cmp x 10-3)
0
20
40
60
80
Tumor - - + + - - + + - - + + - - + +
BAT - + - + - + - + - + - + - + - +
Experiment 1 2 3 4

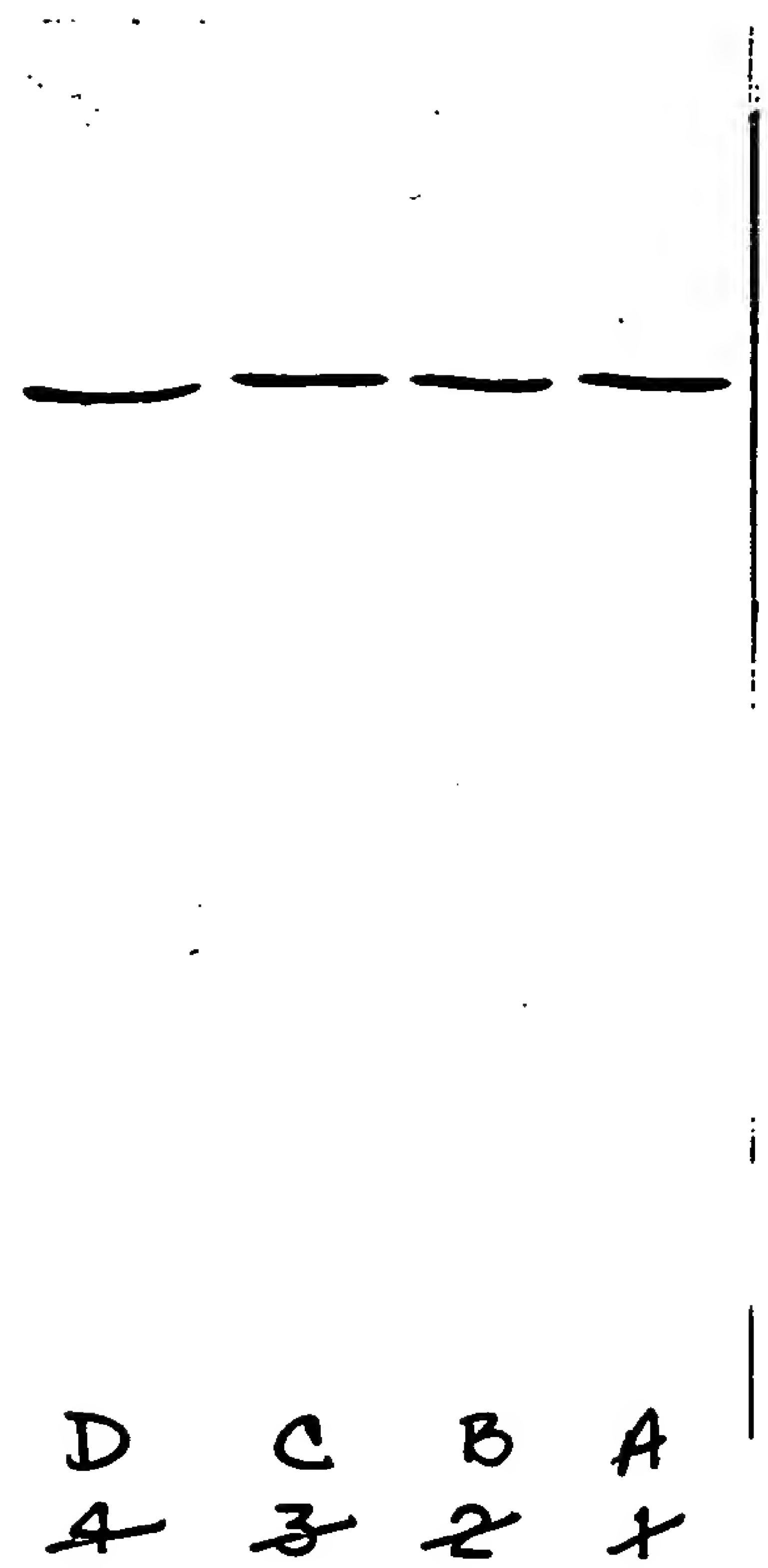


Fig. 4